FEBS Letters 583 (2009) 1544–1547

FEBS Letters
journal homepage: www.FEBSLetters.org

Antifungal properties of a peptide derived from the signal peptide of the HIV-1 regulatory protein, Rev

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ARTICLE INFO

Article history:
Received 6 February 2009
Revised 27 March 2009
Accepted 27 March 2009
Available online 2 April 2009

Edited by Peter Brzezinski

Keywords:
Nuclear entry inhibitory signal peptide of Rev protein (Rev-NIS)
Antifungal peptide
Membrane-active mechanism

ABSTRACT

Antifungal effects of nuclear entry inhibitory signal peptide of HIV-1 Rev protein (Rev-NIS) were investigated. Rev-NIS contained potent antifungal activities without hemolytic effects. To understand the antifungal mechanism(s), *in vivo* and *in vitro* fluorescent studies were conducted. Flow cytometric analysis with bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC$_4$(3)] and calcein-leakage measurement from large unilamellar vesicles (LUVs) indicated that Rev-NIS depolarized and disrupted the fungal membranes. These results were further confirmed by using giant unilamellar vesicles (GUVs). The current study suggests that Rev-NIS exerts its antifungal activity with membrane-active mechanism(s).

1. Introduction

A rapid and radical increase in the emergence of antibiotics-resistant microbes has occurred in recent years [1]. This has not only made treatment for infectious diseases more problematic, but has also resulted in the reappearance of many diseases thought to be under control. To solve these problems, new antibiotics has become inevitable and urgent [2]. Antimicrobial peptides (AMPs) are thought to be novel model regarding therapeutic agents. In contrast to conventional antibiotics, AMPs rapidly permeate and disrupt the cell membrane, causing damage hard to repair. Therefore, microbial resistance may occur with much lower probability than that observed with available antibiotics [3]. At this point in time, development and improvement of novel antimicrobial peptides are indispensable and crucial.

Human immunodeficiency virus type-1 (HIV-1) encoded the regulatory protein, Rev, which is constantly shuttling between the cell nucleus and the cytoplasm in the host cells, under the control of a signal-mediated active import and export system, probably over the support of the NIS (nuclear entry inhibitory signal) function [4–7]. Without NIS, Rev may migrate back through the nuclear pore against the well-controlled nucleo-cytoplasmic shuttling to complete its role in each compartment. Residues 11–20 of the NIS (Rev-NIS, ELLKAVRLIK) especially are comprised of the conserved hydrophilicity motif, which forms an amphipathic helix. Significantly, this motif and its helical structure were thought to be critical regarding the function of NIS as well as Rev [8]. Although nuclear entry inhibitory signal peptide of Rev protein (Rev-NIS) had a noteworthy potential as an antimicrobial peptide, its antimicrobial properties had not yet been studied. In this study, the antifungal activity and its mechanism(s) of Rev-NIS were investigated.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized by the solid phase method using 9-fluorenyl-methoxycarbonyl chemistry [9]. The crude peptides were repeatedly washed with diethylether, dried in a vacuum, and purified using a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19 × 30 cm). The purity of the peptides was checked by an analytical reversed-phase HPLC on an Ultrasphere C_{18} column (4.6 × 25 cm; Beckman, Fullerton, CA, USA). The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization MALDI-mass spectrometer [10].

Abbreviations: Rev-NIS, nuclear entry inhibitory signal peptide of Rev protein; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; DiBAC$_4$(3), bis-(1,3-dibutylbarbituric acid) trimethine oxonol; LUVs, large unilamellar vesicles; GUVs, giant unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.03.063

2.2. Antifungal activity

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were both obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Malassezia furfur* (KCTC 7744) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. Fungal cells were cultured in YPD broth (Difco) with aeration at 28 °C. *M. furfur* was cultured at 32 °C in modified Bacto yeast extract/malt extract (YM) broth (Difco) containing 1% olive oil. Fungal cells (2×10^4 cells/ml) were inoculated into either YPD or YM broth and 0.1 ml/well of the mixture was dispensed into microtiter plates. Minimum inhibitory concentration (MIC) was determined by means of a serial twofold dilution of the peptides, following the micro-dilution method and MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [11]. After 48 h of incubation at either 28 °C or 32 °C, the minimal peptide concentration to prevent the growth of a given test organism was determined and defined as MIC. Growth was assayed with microtiter ELISA Reader by monitoring absorption at 580 nm. MIC values were determined by three independent assays.

2.3. Kinetics of fungal killing

Exponential phased *C. albicans* (ATCC 90028) cells (2×10^6 CFUs/ml) were incubated with either Rev-NIS or melittin (at the MIC). The cultures were obtained and spread on an YPD agar plate, and then the colony forming units (CFUs) were counted after incubation for 24 h at 28 °C [12]. The values represented the average of triplicate measurements in three independent assays.

2.4. Hemolytic activity

The hemolytic activity of the peptides was evaluated by determining the release of hemoglobin from a 4% suspension of fresh human erythrocytes at 414 nm with an ELISA plate Reader [11]. The hemolysis percentage was calculated by using the following equation: Percentage hemolysis = [($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)] × 100 [13].

2.5. Changes of the plasma membrane potential

For analysis of the membrane integrity after the peptide treatment, log-phased cells of *C. albicans* (1×10^8 cells) were harvested and resuspended with 1 ml fresh YPD media, containing either Rev-NIS or melittin (at the MIC). After incubation for 3 h, the cells were washed with PBS. To detect the depolarization of the cell membrane, 1 ml of PBS, containing 50 μg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Molecular Probes, Eugene, OR, USA), was added and then the samples were incubated for 1 h at 4 °C in darkness [14]. Flow cytometric analysis was conducted by means of a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.6. Calcein leakage measurement

Calcein-encapsulated large unilamellar vesicles (LUVs), composed of phosphatidylcholine(PC)/phosphatidylethanolamine(PE)/phosphatidylinositol(PI)/ergosterol (5:4:1:2, w/w/w/w), were prepared by vortexing the dried lipids in a dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA [pH 7.4]). The suspension was frozen-thawed in liquid nitrogen for 11 cycles and extruded through polycarbonate filters (two stacked 100 nm pore size filters) by a LiposoFast extruder (Avestin Inc., Ottawa, Canada). Untrapped calcein was removed by a gel filtration process on a Sephadex G-50 column. The leakage of calcein from the LUVs was monitored by measuring the fluorescent intensity, at an excitation wavelength of 490 nm and an emission wavelength of 520 nm, with a RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). The measurements were conducted at 25 °C. For determination of 100% dye release, 20 μl of 10% Triton X-100 was added to the vesicles. The percentage of dye leakage caused by the peptides was calculated as follows: dye leakage (%) = $100 \times (F - F_0)/(F_t - F_0)$, where F represents the fluorescent intensity achieved by the peptides and F_0 and F_t represent the fluorescent intensities without the peptides and with Triton X-100, respectively [15].

2.7. Formation and microscopic observation of GUVs

Giant unilamellar vesicles (GUVs) were prepared by using ITO (indium tin oxide) glasses. Lipids were prepared at a concentration of 3.75 mg/ml in chloroform. The lipid solutions (100–200 μl) were deposited in a spin coater (Spin Coater, ACE-1020 Series) and the glass was coated for 2 min. Then the coated ITO glass was evaporated under a vacuum for 2 h. The following procedure was used in succession; a square frame was created from silicon served as a thickness (2 mm) spacer between the lipid-coated glass and normal glass. The chamber was filled with 10 mM HEPES buffer (pH 7.2) through a hole in the silicon spacer. Immediately, the application of 1.7 V (peak-to-peak, sine wave) and 10 Hz to the ITO electrodes was made by using a sweep function generator (Protek, SWEEP FUNCTION GENERATOR 9205C) for 2 h. GUVs from the ITO glass were then detached in conditions of 4 V (peak-to-peak) and at 4 Hz for 10 min. Rev-NIS (at the MIC) was treated and changes of GUVs were observed by using an inverted fluorescence phase contrast microscope (Leica, DFC420C).

3. Results and discussion

Peptides were chemically synthesized and the antifungal effects of Rev-NIS were investigated. In this study, melittin was used as a positive control. Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) is the well-known antimicrobial peptide from the venom of the honey bee *Apis mellifera* [16]. It was suggested that melittin formed pores and translocated the membrane [17]. The result showed that the fungal strains, as tested, were susceptible to Rev-NIS, with MIC values in 10–40 μM range, while melittin had determined at MIC values of 2.5–5 μM (Table 1). Furthermore, the kinetic fungal killing assay conducted with *C. albicans* indicated that the antifungal activity of Rev-NIS might be due to cidal, rather than static action (Fig. 1). Although most antimicrobial peptides exhibited good cell selectivity among microbial and mammalian host cells, some antimicrobial peptides showed lytic activities toward mammalian cells [13]. Therefore, we investigated the hemolytic effects of Rev-NIS as an indicator of its cytotoxicity to mammalian cells. The result showed that Rev-NIS did not cause hemolysis on human erythrocytes, while melittin induced much more potent hemolytic activities at most tested concentration levels (Table 2). Although Rev-NIS exhibited less potent antifungal activity than melittin, which showed both potent antifungal and hemolytic activity, these

Table 1
Antifungal activity of Rev-NIS.

Peptide	MIC (μM)			
	C. albicans	*C. parapsilosis*	*M. furfur*	*T. beigelii*
Rev-NIS	10	10	20	20–40
Melittin	2.5	2.5	5	5

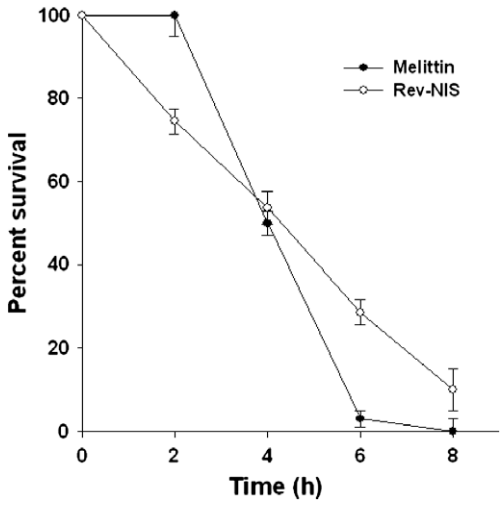

Fig. 1. Time killing plots for *C. albicans* by 10 μM of Rev-NIS or 2.5 μM of melittin. The error bars represent the standard deviation (S.D.) values for three independent experiments, performed in triplicate.

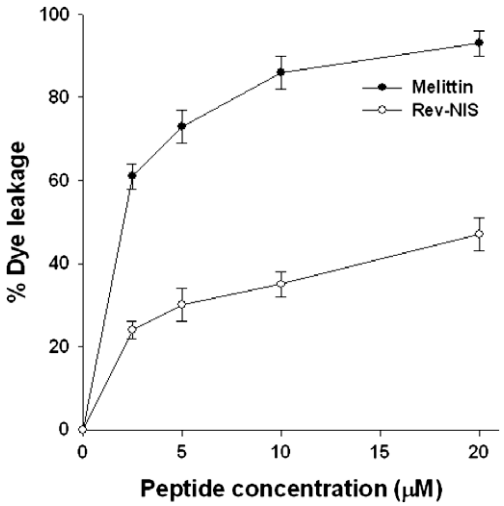

Fig. 3. Calcein leakage from LUVs in the presence of various concentrations (2.5, 5, 10, 20 μM) of the peptides. The error bars represent the standard deviation (S.D.) values for three independent experiments, performed in triplicate.

Table 2
Hemolytic activity of Rev-NIS against human erythrocytes.

Peptide	% Hemolysis (μM)						
	100	50	25	12.5	6.25	3.13	1.56
Rev-NIS	0	0	0	0	0	0	0
Melittin	100	100	100	100	100	98	98

results demonstrated that Rev-NIS exhibited good cell selectivity between fungal cells and mammalian cells. Also, it was suggested that Rev-NIS could be applied to therapeutic agents regarding fungal diseases in humans.

To understand the mechanism(s) of Rev-NIS for exerting antifungal activities, *C. albicans*, both a commensal and opportunistic pathogen of ever-increasing medical importance [18], was selected. To investigate whether Rev-NIS can affect *C. albicans*, the potential dissipation of the plasma membrane was examined by staining with $DiBAC_4(3)$, a translational membrane potential dye [19,20]. $DiBAC_4(3)$ contains a high voltage sensitivity and can enter depolarized cells, where it binds to lipid-rich intracellular components [14]. The result showed that Rev-NIS caused significant accumulations of $DiBAC_4(3)$ (Fig. 2). This indicated that Rev-NIS affected fungal cells by damaging their membranes, thus disrupting the fungal membrane potential.

Finally, to confirm the membrane-disruptive mechanism(s) of Rev-NIS, the effects of Rev-NIS on calcein-entrapped LUVs and GUVs containing rhodamine-conjugated PE were examined, which were composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), mimicking the membranes of *C. albicans* [21]. The calcein leakage percentage was measured to assess its ability to permeabilize the membrane. GUVs, of which the diameters exceed 10 μm, have been used for physical and biological investigations, such as the elastic properties of phospholipid membranes [22,23], the changing shapes of vesicles [24–27], the interaction of cytoskeleton proteins with the membranes [28], membrane fusion [29], and the reconstitution of artificial cells [30,31]. These studies are useful in understanding the dynamics of biomembranes [32]. The result showed that Rev-NIS significantly disrupted the liposome and caused calcein-leakage from the LUVs (Fig. 3). Also, rhodamine concentrations of the GUV progressively decreased after the addition of Rev-NIS (Fig. 4). These indicated that Rev-NIS exerted dye-leakage from liposomes due to disruption of the fungal model membranes. These results may also confirm the membrane-active mechanism of Rev-NIS.

In this study, the antimicrobial effects of Rev-NIS were investigated. Although the exact mechanism(s) of Rev-NIS have not been fully elucidated, this study suggests that Rev-NIS contains considerable antifungal activities for regarding disruption of the fungal plasma membranes.

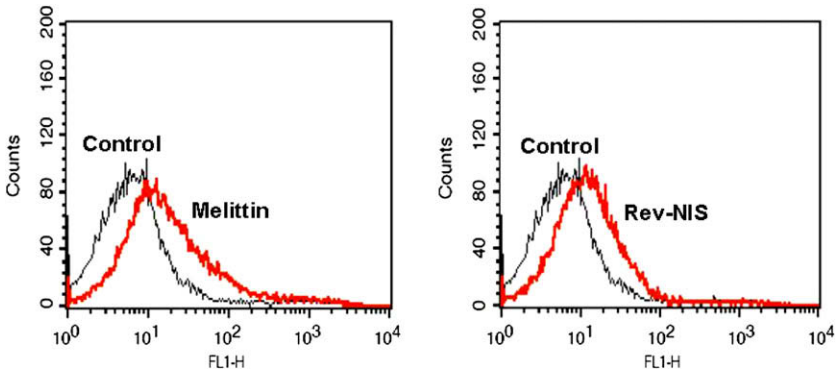

Fig. 2. FACScan analysis of $DiBAC_4(3)$ staining in *C. albicans*. Histograms showed the fluorescent intensity of stained $DiBAC_4(3)$ in *C. albicans* after treatment of 10 μM of Rev-NIS or 2.5 μM of melittin.

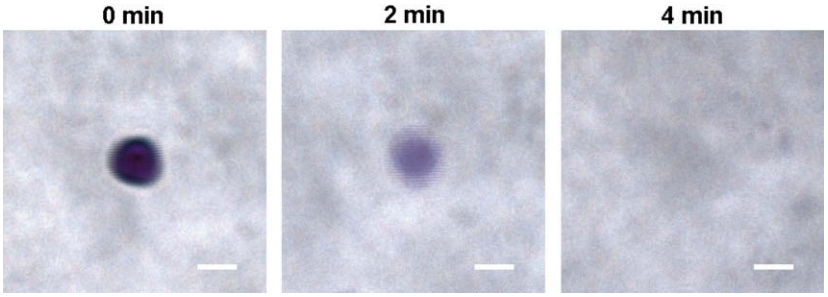

Fig. 4. Phase contrast images of GUVs, labeled with rhodamine, induced by 10 μM of Rev-NIS. The times above each image show the time after the addition of peptide. The bar corresponds to 10 μm.

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